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Advanced Glycation End Products (AGEs) Co-Localize with AGE Receptors in the Retinal Vasculature of Diabetic and of AGE-Infused Rats

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Advanced glycation end products (AGEs), formed from the nonenzymatic glycation of proteins and lipids with reducing sugars, have been implicated in many diabetic complications; however, their role in diabetic retinopathy remains largely unknown. Recent studies suggest that the cellular actions of AGEs may be mediated by AGE-specific receptors (AGE-R). We have examined the immunolocalization of AGEs and AGE-R components R1 and R2 in the retinal vasculature at 2, 4, and 8 months after STZ-induced diabetes as well as in nondiabetic rats infused with AGE bovine serum albumin for 2 weeks. Using polyclonal or monoclonal anti-AGE antibodies and polyclonal antibodies to recombinant AGE-R1 and AGE-R2, immunoreactivity (IR) was examined in the complete retinal vascular tree after isolation by trypsin digestion. After 2, 4, and 8 months of diabetes, there was a gradual increase in AGE IR in basement membrane. At 8 months, pericytes, smooth muscle cells, and endothelial cells of the retinal vessels showed dense intracellular AGE IR. AGE epitopes stained most intensely within pericytes and smooth muscle cells but less in basement membrane of AGE-infused rats compared with the diabetic group. Retinas from normal or bovine-serum-albumin-infused rats were largely negative for AGE IR. AGE-R1 and -R2 co-localized strongly with AGEs of vascular endothelial cells, pericytes, and smooth muscle cells of either normal, diabetic, or AGE-infused rat retinas, and this distribution did not vary with each condition. The data indicate that AGEs accumulate as a function of diabetes duration first within the basement membrane and then intracellularly, co-localizing with cellular AGE-Rs. Significant AGE deposits appear within the pericytes after long-term diabetes or acute challenge with AGE infusion conditions associated with pericyte damage. Co-localization of AGEs and AGE-Rs in retinal cells points to possible interactions of pathogenic significance. (Am J Pathol 1997, 150:523–531)

The nonenzymatic reaction of sugars with proteins through the Maillard reaction ultimately leads to the formation of advanced glycation end products (AGEs).[1] These diverse and highly reactive protein adducts have been shown to accumulate in animal and human tissues with aging and at an accelerated rate in diabetes.[2] AGEs are responsible for many pathophysiological effects, such as cross-link formation in the extracellular matrix,[3] mediating overproduction of basement membrane (BM) components,[4] interference with nitric oxide (NO)-mediated vasodilation,[5,6] and vascular permeability.[6]

Through *in vitro* and *in vivo* studies it is clear that AGEs play an important role in diabetic nephropathy[1–7] and atherosclerotic vasculopathy.[1–3,6,8,9] At present it remains equivocal whether AGEs are a factor in the pathogenesis of diabetic retinopathy. A receptor-mediated AGE toxicity to retinal pericytes has been demonstrated *in vitro*,[10] whereas Hammes et al[11] have observed that administration of the AGE inhibitory drug aminoguanidine to diabetic rats prevents pericyte loss in the retinal vessels. However, it has yet to be demonstrated whether the retinal vas-

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cular cells bind or interact specifically with AGEs and it remains controversial whether AGEs have any direct role in retinal vascular BM thickening, pericyte loss, and capillary closure, lesions that are among the pathological hallmarks of preproliferative diabetic retinopathy.[10–12]

The existence of specific AGE receptors (AGE-Rs) that can modulate cellular responses through a receptor-ligand interaction provides a mechanism to explain not only accumulation of AGEs in many cell types but also abnormal cellular responses.[8,13–18] Recently, several components of this AGE-R system have been characterized.[19–21] Two of these membrane proteins, a ~50- to 60-kd protein,[20,21] identical to a component of the oligosaccharyltransferase-48 complex (OST-48),[22] and a ~90-kd protein,[20,21] identical to 80 K-H,[23] a protein kinase C substrate, hereafter referred to as AGE-R1 and AGE-R2, respectively, have been found on numerous cells and tissues including phagocytic, vascular, and renal cells.[21]

In the current study, we have investigated the temporal appearance of AGEs and of two components of the AGE-R system, R1 and R2, in the retinal vasculature of normal diabetic and normal rats challenged briefly with exogenous AGE-albumin.

Materials and Methods

Animals

Experimental diabetes was induced in male Wistar albino rats (250 g; n = 12) by a single injection of streptozotocin (45 mg/kg intraperitoneally). Fasting blood glucose in these animals, tested monthly, was 30 to 50 mmol/L. All animals were fed regular rat chow and water *ad libitum*; after an initial loss of weight (<10%), diabetic animals continued to gain weight. No loss of weight was noted in the AGE-infused or control rats. Diabetic animals were sacrificed after 2, 4, and 8 months duration of diabetes, together with age- and sex-matched controls (n = 6). In addition, groups of age- and sex-matched normoglycemic rats (250 g; n = 12/group) were given injections of unmodified bovine serum albumin (BSA) or AGE-BSA, 40 mg/kg/day, intravenously, for 14 days.[4,6,7] AGE levels in serum of diabetic animals and normal animals were analyzed using a standardized AGE enzyme-linked immunosorbent assay (ELISA; diabetic, 52 ± 12 U/ml; normal, 35 ± 7 U/ml).[24] In AGE-infused rats, treatment resulted in sustained two- to three-fold increases in serum AGE when compared with native albumin injection (72 ± 12 U/ml and 28 ± 6 U/ml, respectively).[4,6,7] Animals were sacrificed either 1 to 2 hours (n = 6) or 2 days (n = 6) after the final injection, allowing sufficient time for serum AGE to return to preinfusion normal levels.

Preparation of Advanced Glycation End Products

BSA (low endotoxin, fraction V; Sigma Chemical Co., St. Louis, MO) was passed over an affi-Gel Blue column (Bio-Rad, Melville, NY), a heparin-Sepharose CL6B column (Pharmacia, Piscataway, NJ), and an endotoxin-binding affinity column (Pierce, Rockport, IL) to remove possible contaminants. BSA modified by advanced glycation was prepared by incubation in 0.5 mol/L glucose for 6 weeks as previously described,[4,6,7] and AGE levels were measured by an AGE ELISA[24] (AGE-BSA, 250 AGE U/mg protein; unmodified BSA, 0.9 AGE U/mg protein). Each reagent contained <0.2 ng/ml of endotoxin (E-Toxate, Sigma).

Production of Antibodies

A well characterized polyclonal anti-AGE-RNAse antibody[24,25] and a monoclonal anti-AGE antibody, kindly provided by Dr. H. Founds (Alteon, Northvale, NJ), were used in these studies, and both antibodies yielded identical results (only anti-AGE-RNAse is shown, unless otherwise specified). Polyclonal antisera against recombinantly produced AGE-R components, OST-48 (AGE-R1) and 80 K-H (AGE-R2),[21] were used for the visualization of these epitopes on rat retinal cells. Immune and preimmune IgG fractions were purified by protein-G columns (Pharmacia) as previously described.[21] The specificity of these antibodies has been confirmed by Western blot and flow cytometry, and titers of each antibody to the recombinant proteins were above 1:10,000 as estimated by a conventional ELISA.[21]

Tissue Processing and Immunolocalization

For preparation of retinal digests, the eyes were removed and placed immediately in 10% buffered formalin overnight. The anterior segment and lenses were removed and the neural retina was separated from the eye-cup. Each retina was then digested in 3% trypsin as previously described.[26] The entire retinal vascular tree was then washed in distilled water before mounting to a Silane-Prep slide (Sigma).

Posterior segments were also processed by immersion in OCT compound and snap-freezing in vis-

cous isopentane (cooled with liquid nitrogen). Fresh-frozen sections were cut at 8 μm, fixed in cold acetone for 20 minutes, and stored at −20°C.

For immunocytochemistry, the retinal digests were hydrated in phosphate-buffered saline (PBS) containing 0.1% BSA, 0.01% Triton X-100, and 0.01% sodium azide, and before addition of the primary antibody, they were incubated in 10% normal goat serum (20 minutes). Control digests were incubated in a similarly diluted mouse or rabbit IgG or preimmune serum at the primary antibody stage. For preabsorbed controls, optimally diluted antibodies to AGE were mixed with an equal volume of 100 μg/ml AGE-BSA for 1 hour before incubation. After incubation with the primary antibody (range, 1:200 to 1:2000 according to antibody) for 18 hours at 4°C, the slides were washed (three times for 10 minutes each in PBS) and then exposed to the appropriate biotinylated secondary antibody (1:200; Amersham, Arlington Heights, IL) for 1 hour, washed again, and incubated with streptavidin fluorescein isothiocyanate (Amersham) for an additional hour. After further washing, slides were mounted in 50% aqueous glycerol and photographed on a fluorescence microscope. To assess the retinal changes, the coverslips were then gently removed from the slides and the digests were washed in ascending ethanols and stained with hematoxylin and periodic acid-Schiff. The number of acellular capillaries was determined by counting the number of acellular strands (mean of 12 fields of view per specimen at ×250 magnification) in a blinded fashion.[26] The standard deviation from the mean was calculated and comparisons between groups were carried out using a Student's two-tailed *t*-test.

Results

Acellular Capillary Estimations

Gross pathological examination of retinas from 2- and 4-month-diabetic groups showed no significant areas of cell loss, capillary closure, or other vascular abnormalities. However, the retinal vessels of rats with 8 months of diabetes showed areas of capillary closure, characterized by the absence of pericyte and/or endothelial cell nuclei (Figure 1; $P < 0.01$). The retinas from AGE-BSA-infused rats, showed some areas with acellular capillaries; however, these did not reach statistical significance within the 2-week treatment period ($P < 0.07$). Occasional acellular capillary strands were also present in some segments of normal retina. The acellular capillary estimates for each of the experimental groups are presented in Figure 1.

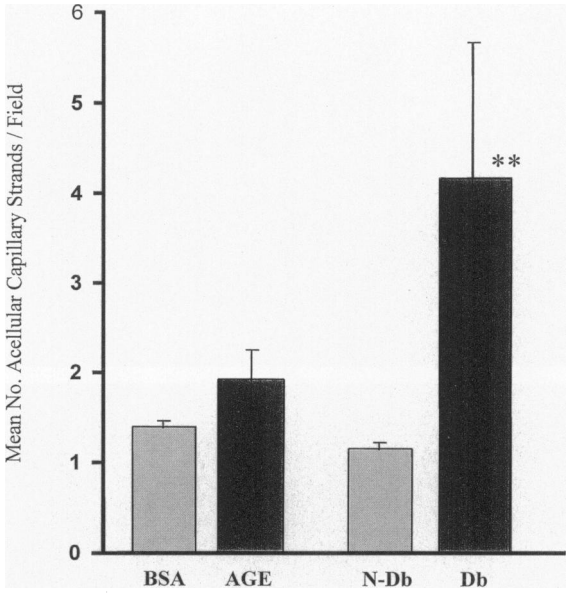

Figure 1. *Acellular capillaries in normal (N-Db), 8-month STZ-diabetic (Db), and BSA- and AGE-infused rats.* ***Diabetic retinae* versus *nondiabetic controls* ($P < 0.01$); *BSA* versus *AGE-infused animals* ($P < 0.07$). *Data are expressed as the mean* (±*SD*) *number of acellular strands* ($n = 6$ *per group*).

AGE Immunolocalization

Normal rat retinal vessels were uniformly negative for AGE immunoreactivity (IR), and only a background IR was observed (Figure 2A); however, after 2 months of diabetes, the retinal vasculature showed a comparative increase in diffuse AGE IR (Figure 2B). At 4 months of diabetes, AGE IR was more extensively distributed within the capillary network, becoming more visible intracellularly (Figure 2C). At 8 months of diabetes, the retinal capillaries showed intense AGE IR within pericytes and endothelium, whereas in the retinal arteries, the smooth muscle cells were also intensely positive (Figure 2, D and E). Retinal digests of 8-month-diabetic rats stained using preabsorbed anti-AGE antibodies showed a low level of IR (Figure 2F). In cross section, retinal vessels demonstrated sparse AGE IR in BM, although the cellular localization of AGEs from this viewpoint was difficult to determine accurately.

We subsequently compared the distribution of endogenous AGE IR to that induced by intravenously administered exogenous AGEs in retina of nondiabetic rats after a 2-week infusion. In concordance with previous data,[6,7] serum AGE levels rose 2.5-fold in the AGE-infused animals above the control (BSA-infused) group (data not shown). Within 2 hours from

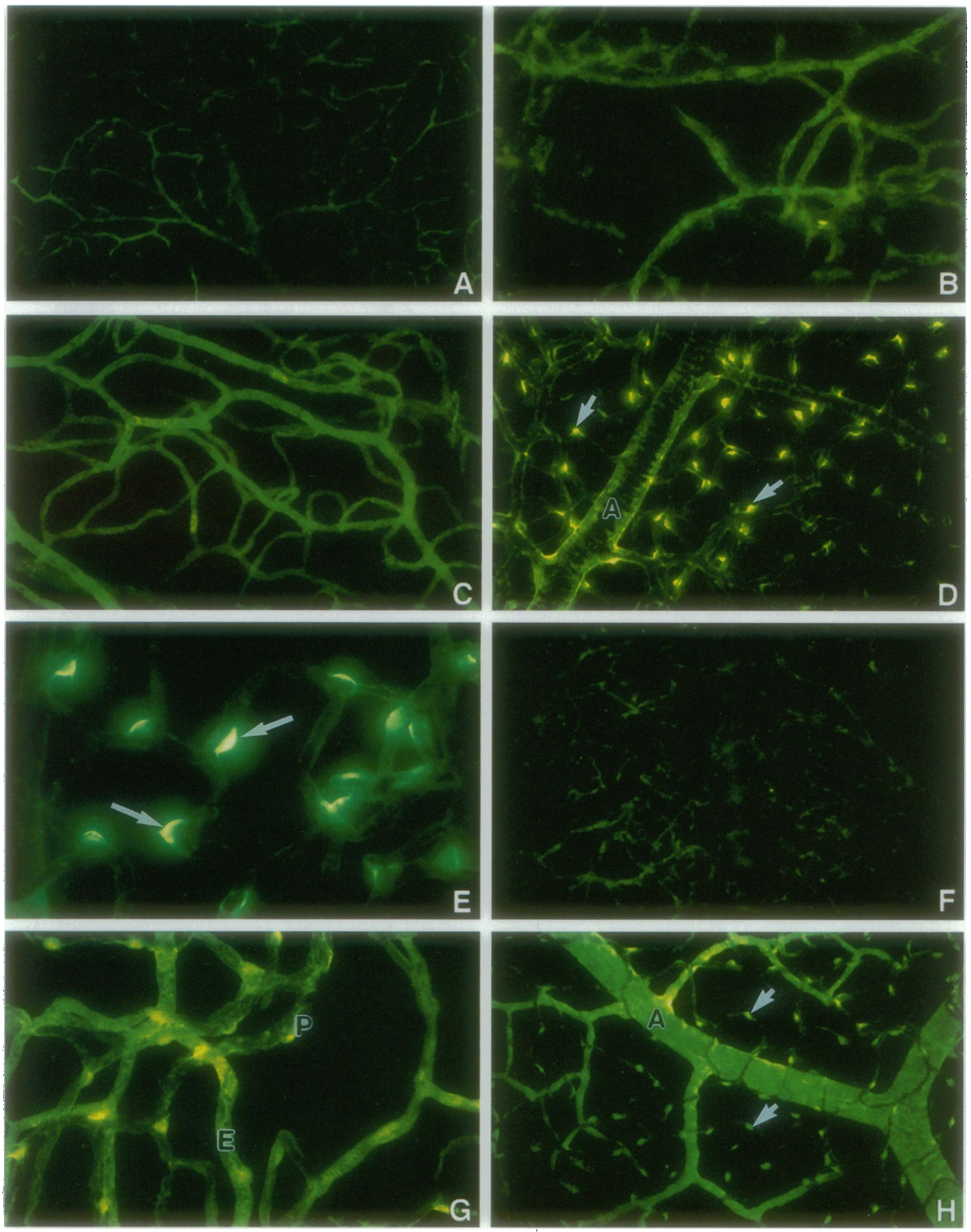

Figure 2. *AGE immunoreactivity (IR) of rat retinal vessels isolated by trypsin digestion.* A: *Normal rat retina vascular digest. Magnification, ×100. The retinal vessels display background AGE IR,* B: *Two-month diabetic retina showing sparse AGE IR that appears largely associated with BM. ×200.* C: *Four-month diabetic retina in which the vessels show a comparative increase in AGE IR. ×200.* D: *Eight-month diabetic retina showing AGE IR with smooth muscle cells of the retinal artery and arterioles (A) as well as the pericyte cells of the capillaries (arrows). ×100.* E: *Higher magnification of eight-month diabetic retinal capillary bed in which the pericyte cells are strongly immunofluorescent. ×400.* F: *Eight-month diabetic retina stained using anti-AGE IgG preabsorbed with AGE-BSA. ×100.* G: *Fourteen-day AGE-infused rat in which the retina was fixed 2 hours after the last infusion. AGE IR is associated with BM, endothelium, and pericyte cells of the capillaries. ×400.* H: *Fourteen-day AGE-infused rat in which the retina was fixed 2 days after the last infusion. ×200. AGE IR is associated largely with the retinal arteries and arterioles and pericytes of the capillaries. Data are representative from six animals per group.*

the last AGE injection, AGE IR was intense within the endothelial cells and pericytes of the capillary beds and the endothelial and smooth muscle cells of the small arteries and arterioles (Figure 2G). At 2 days after the last AGE injection, while the vascular endothelium exhibited diminished AGE IR, there was still intense AGE staining within the pericytes of capillaries and veins and the smooth muscle cells of arteries and arterioles (Figure 2H). Compared with the strongly positive BM of 4- and 8-month-diabetic retina, the BM in these animals was only minimally AGE immunoreactive (Figure 2H). The retinas of normal rats infused with nonglycated BSA remained negative for AGE IR. Also, retinal specimens from AGE-infused rats stained with primary anti-AGE antibody preabsorbed with excess AGE-BSA showed only background IR, comparable with Figure 2F (not shown). Lastly, the pattern of AGE localization shown with the monoclonal anti-AGE monoclonal antibody was identical to that obtained using the polyclonal anti-AGE antibody (data not shown).

AGE Receptor Immunolocalization

Two of the currently known AGE-R components, AGE-R1 and -R2, were examined by immunoreagents raised to the recombinantly made proteins.[21] In either normal, AGE-infused, or diabetic rat retinas, immunostaining for AGE-R showed an identical distribution pattern, mostly confined to the pericytes and smooth muscle cells and to a lesser degree to endothelial cells (Figure 3, A and B). Both receptor components appeared to co-localize with intracellular AGEs, although neither the pattern nor the intensity of fluorescence appeared to vary with presence of hyperglycemia or exogenous AGEs. Retinas stained with preimmune sera showed only background IR (Figure 3, C and D).

In retinal cross sections, endothelial cells and smooth muscle cells of the retinal arteries as well as endothelial cells and pericytes of the capillary beds (inner and outer plexiform layers) showed positive IR to AGE-R1 and AGE-R2, as did the photoreceptor cell bodies in the outer retina (Figure 3, E and F). AGE-R2 stained strongly at the nerve fiber layer, indicating that this protein was also present in ganglion cells. Consistent with other retinal layers, the large-vessel endothelial cells and, in particular, smooth muscle cells of the choroid also demonstrated positive IR for both receptors. By contrast, the choriocapillaries was largely AGE IR negative. An overall comparison of AGE-R staining between retinal sections of normal control, diabetic, and AGE-infused animals showed no significant difference in intensity or distribution.

Discussion

The current investigation demonstrates that sustained hyperglycemia results in a gradual AGE accumulation within retinal cellular and extracellular structures, including BM, endothelium, pericytes, and smooth muscle cells of diabetic rats. AGE epitopes occur first in the retinal vascular BM of newly diabetic rats (<2 months) and increase progressively until, by 8 months of diabetes, prominent AGE IR within retinal vascular cells, especially pericytes, co-exists with established retinal changes.

It has been previously reported that cell loss and acellular capillaries appear after 6 to 8 months duration of diabetes in the rat.[27,28] Consistent with these reports, we observed no significant difference in the frequency of these lesions up to 8 months of diabetes.

It has now been established unequivocally that sustained hyperglycemia is critical for the development of retinopathy.[29,30] Among the currently debated hypotheses, the late nonenzymatic glycation product formation has been suggested as a plausible factor for the irreversible components of diabetic retinopathy.[1,2,11]

In this study, besides the BM, AGE-like epitopes featured within the retinal vascular cells, especially after prolonged hyperglycemia. The appearance of AGEs within the BM before their accumulation within the pericytes indicated that the rate of extracellular AGE formation may need to exceed the rate of its removal by cellular mechanisms, beyond which cell toxicity ensues. Previous *in vivo* studies have demonstrated autofluorescent AGEs in association with the BM of diabetic rat retinal vessels,[11] whereas AGE inhibition by aminoguanidine prevented pericyte loss and microaneurysm formation[11,31] and protected against blood-retinal barrier breakdown in diabetic rats.[32] The covalent attachment of reactive AGEs to components of retinal arteries and arterioles may contribute significantly to BM thickening, an established lesion of diabetic vasculopathy,[33,34] and eventually to other forms of AGE-mediated toxicity, as shown in other tissues, eg, skin and kidney.[15,35,36] These studies have pointed to a causal link between glycation and microvascular disease; however, the temporal relationship between AGE formation and development of diabetic retinopathy has not been easy to determine, nor have the interactions of retinal cells with AGEs been investigated independent of diabetes.

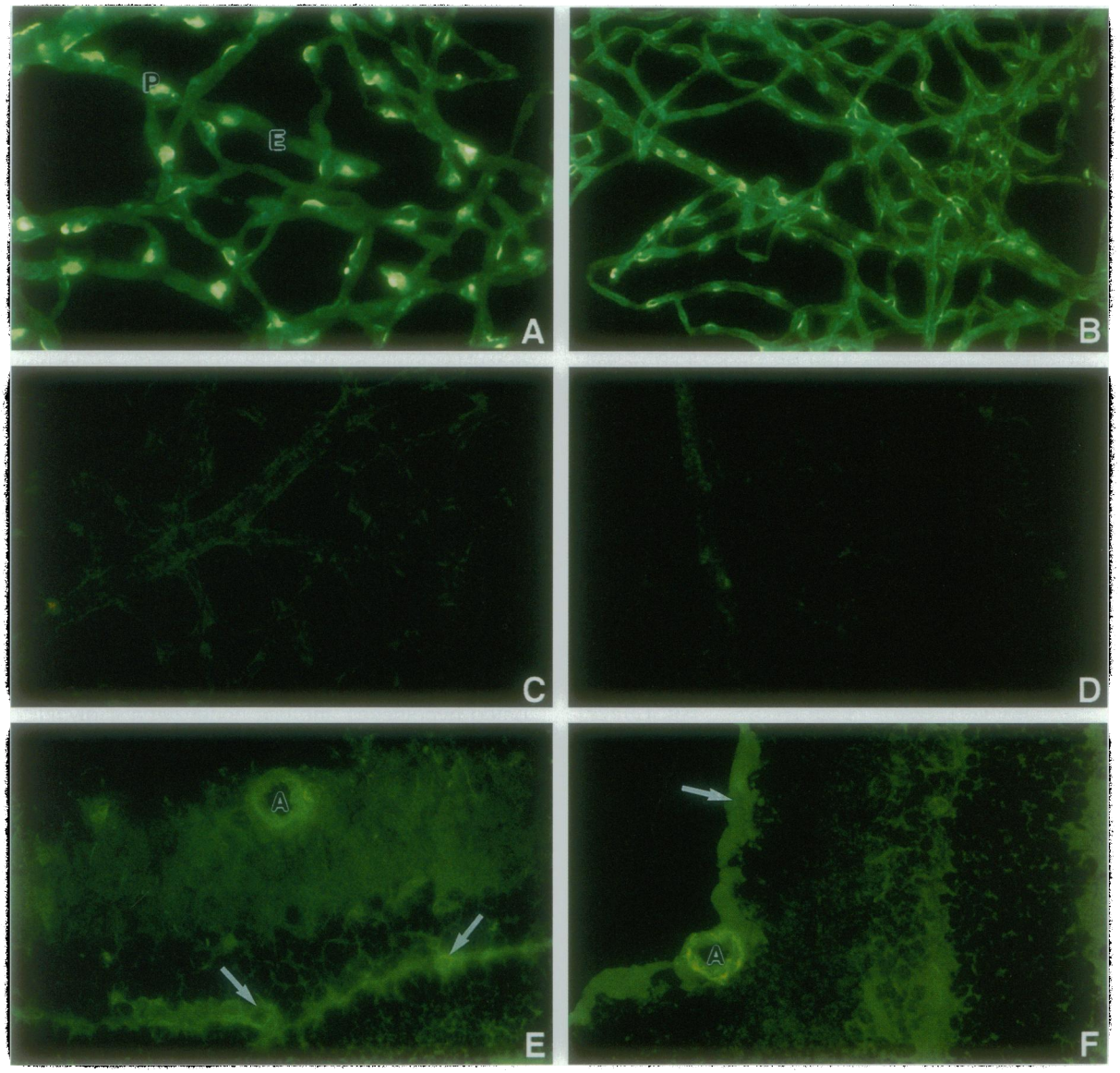

Figure 3. *AGE-R distribution on normal and STZ-diabetic rat retina.* **A:** *AGE-R1 in normal rat retinal vasculature in which the endothelium and pericytes are stained prominently. Magnification, ×200.* **B:** *AGE-R2 in normal rat retinal vasculature showing a similar localization pattern to* **A**. *×100.* **C:** *Normal rat retinal vasculature stained using AGE-R1 preimmune serum. ×200.* **D:** *Eight-month diabetic rat stained with AGE-R2 preimmune serum. ×100.* **E:** *Frozen cross section of whole diabetic rat retina showing AGE-R1 IR. The retinal capillaries in the outer plexiform layer* (**arrows**) *and a retinal artery (A) in the nerve fiber layer show positive staining. ×200.* **F:** *Frozen cross section of whole diabetic rat retina in which a retinal artery (A) and nerve fiber layer* (**arrow**) *are stained positive for AGE-R2. ×200. Data are representative from six animals per group.*

To understand the topography of AGE deposition before the development of diabetic retinal damage, we explored AGE localization within the retina after the infusion of AGEs for a period brief enough so as not to cause overt toxicity. It has been previously demonstrated that AGE infusion for periods longer than 4 weeks leads to excessive production of glomerular extracellular matrix components[4] and vascular dysfunction, manifested as increased vascular permeability,[6] adhesion molecule over-expression,[8] and impaired vasodilatory responses.[6] Longer periods of AGE administration have been shown to promote diabetic nephropathy-like glomerular lesions and albuminuria in normal animals.[7] Thus, the brief 2-week-long AGE treatment produced no overt changes to the retinal vasculature. The retinal endothelium appeared to rapidly internalize circulating AGE-albumin from the intravascular space and to process them without retaining them. In contrast, processing of AGEs by pericytes and smooth muscle cells was delayed for at least 2 days after the termination of the infusion, raising questions about

the mechanisms for processing AGE substances by each cellular system. This, however, is still to be defined.

Tissue-specific effects of AGEs are mediated through interactions with AGE-Rs identified in several tissues, including vascular endothelium and smooth muscle cells.[14–16,18] Based on previous evidence indicating that endothelial cell uptake, transcytosis, and/or degradation of AGEs occurs *via* these receptors,[18] it can be assumed that the appearance of intracellular AGEs was, at least in part, due to uptake by these receptors. The results obtained from the AGE infusion also suggest that retinal endothelium is capable of endocytosing AGEs directly from the bloodstream *via* receptors located at the luminal surface of the endothelial monolayer. Moreover, the data show that blood-borne AGEs can successfully cross the blood-retina barrier. This apparently leads to AGE epitope presentation to and internalization by adjacent pericytes, as indicated by their densely positive AGE immunostaining after AGE infusion. Thus, although the retinal endothelium does not retain these modified proteins, it may permit their transfer and their release to subendothelial tissues, pericytes, and smooth muscle cells. Under nonpathological conditions, the retinal endothelium constitutes a significant barrier as well as a system for transporting selected macromolecules *via* receptor- or non-receptor-mediated pathways.[12,37] Excessive levels of AGE-modified molecules, as they occur in diabetes or renal disease, may lead to barrier breakdown and dysfunctional retinal endothelium, similar to that observed systemically.[6] This can contribute to the mass transcytosis and leakage of proteins toward the retinal extravascular space associated with diabetes.[12] The identification of two known AGE-R components (AGE-R1 and R2) on the retinal endothelium is suggestive of an active role for this system in the microvascular homeostasis; its exact nature, however, remains to be explored.

AGE-R1 and -R2 were also prominently evident on pericytes and smooth muscle cells of arteries and arterioles. The localization pattern of these receptors was similar among the different cell types and did not vary significantly with induction of diabetes or infusion of AGEs in normal rats. Pericytes have been known targets of diabetes-related toxicity,[37,38] and *in vitro* exposure of retinal pericytes to AGEs has led to cellular toxicity.[10] In addition, smooth muscle cell number is reported to decline in retinal arteries and arterioles after long-standing diabetes.[39] The preferential appearance of intracellular AGE deposits within those cells that are most susceptible to toxicity in diabetic retinopathy, together with the simultaneous occurrence of AGE deposits and retinal pathology, are indeed intriguing; however, additional work is needed to establish firmly whether AGEs are direct contributors in retinal cell dysfunction and/or death. In the current investigation, we have demonstrated that retinal vascular cells express AGE-Rs under normal or diabetic conditions, an observation likely to lead into the mechanisms of processing of these ubiquitous substances. The *in vivo* identification of large AGE deposits within retinal vascular cells exposed to either prolonged hyperglycemia or to exogenous AGE administration lends evidence to the growing hypothesis that these reactive adducts can damage retinal vascular cells in susceptible diabetic individuals independently of hyperglycemia.

Acknowledgments

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